THE COMPARISON OF COLONIZATION DYNAMIC OF *Vibrio cholerae* VACCINE CANDIDATES *IN VIVO* AND *IN VITRO*

by

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Dissertation submitted as partial fulfillment for the Degree of Bachelor of Health Sciences (Biomedicine)

March 2008

CERTIFICATE

This is to certify that dissertation entitled "**The Comparison of Colonization Dynamic of *Vibrio cholerae* Vaccine Candidates *In Vivo* and *In Vitro***" is the bonafide record of research work done by Ms Tan Zi Ning during the period from July 2007 to March 2008 under my supervision.

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Date: 26/3/2008

ACKNOWLEDGEMENTS

First of all, I would like to give thanks to Sri Sathya Sai Baba for His blessings and guidance that helped me in reaching the completion of my research project.

Besides, I would like to express my highest appreciation to my supervisor Associate Professor Dr. M. Ravichandran, for giving me the opportunity to do my research and also for his guidance and advices throughout the project.

Next, I gratefully acknowledge my senior, Mr Tan Gim Cheong for his patience in guiding, teaching and helping me throughout my research project.

I would like to thank Mr Kurunathan and other laboratory members for their teaching and help.

I also wish to send my warmest thank to all biomedicine students for sharing their knowledge, help and support.

Last but not least, my special thanks go to my dearest parents and my brother for giving me their steadfast love, encouragement and support to finish my research project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PERBANDINGAN DINAMIK KOLONISASI BAGI STRAIN VAKSIN *Vibrio cholerae* SECARA *IN VIVO* DAN *IN VITRO*

ABSTRAK

Vibrio cholerae merupakan mikroorganisma gram negatif yang menyebabkan penyakit taun. Penyakit taun ialah penyakit cirit-birit yang teruk dan berkeupayaan meragut nyawa pesakit jika pesakit tidak mengambil rawatan dengan segera. Pelbagai jenis vaksin telah dibangunkan dengan menggunakan *V. cholerae* yang mati dan yang dilemahkan. Namun, tahap keberkesanan vaksin ini masih tidak konsisten dan tidak dapat memberi perlindungan sepanjang umur. Selain itu, terdapat vaksin di pasaran yang masih tidak dapat digunakan untuk mencegah kumpulan O139 *V. cholerae*, yang merupakan kumpulan yang paling bahaya. Maka, dalam kajian ini, beberapa strain vaksin bagi *Vibrio cholerae*, yang didapati daripada kumpulan O139 *Vibrio cholerae* dengan adanya mutasi pada beberapa gen toksinnya digunakan untuk membelajari patogenesis penyakit taun. Kajian sitotoksisiti dijalankan untuk mengesahkan peranan toksin RTX yang menyebabkan sel HEp-2 menjadi bulat. Tambahan pula, SDS-PAGE dan Western Blot dijalankan untuk pengesahan lanjutan terhadap ciri-ciri toksin RTX yang menyebabkan depolimerisasi terhadap aktin dan penyilangan antara monomer aktin. VCUSM 9P, VCUSM 11P, VCUSM 14P dan VCUSM 17P menyebabkan kesan bulatan pada sel HEp-2, depolimerisasi terhadap aktin dan penyilangan antara monomer aktin. VCUSM 10p yang mempunyai mutasi pada kedua-dua gen *rtx A* dan *C*, tidak menunjukkan kesan toksin RTX terhadap sel HEp-2. Selain itu, untuk menkaji patogenesis penyakit taun, kedua-dua kajian kolonisasi *in vivo* dan *in vitro* dijalankan dan keberkesanan kolonisasi bagi bakteria dalam kedua-dua kajian dibandingkan. Keputusan menunjukkan kolonisasi bagi strain vaksin *V. cholerae* dalam kajian *in vitro* lebih berkesan daripada kajian kolonisasi *in vivo.*

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ABSTRACT

Vibrio cholerae is a Gram-negative bacterial pathogen that can cause cholera. Cholera is characterized by a severe watery diarrhea and it is a life-threatening diarrheal disease that eventually kills the victims within hours of the onset of symptoms if not treated on time. There are many killed and live-attenuated vaccines developed for *V. cholerae*, but the efficacy of these vaccines varies and does not give life long protection. However, there is still no commercially available vaccine for O139 *V. cholerae* serogroup, which is the most virulent strain. Thus, in this study, several *V. cholerae* vaccine candidates which were derivatives of the O139 *Vibrio cholerae* serogroup with the mutation on different virulence factors were used to study the cholera pathogenesis. The cytotoxicity assay was carried out to confirm the role of RTX toxin which causes the rounding effect on the HEp-2 cells. SDS-PAGE and Western Blot were carried out to further examine the RTX toxin property in causing depolymerization of actin and cross-linking of actin monomers. VCUSM 9P, VCUSM 11P, VCUSM 14P and VCUSM 17P caused rounding of the HEp-2 cells, depolymerization of actin and cross-linking of actin monomers. VCUSM 10P which has mutation on both *rtx A* and *C* genes showed no effect of the RTX toxin to the HEp-2 cells. In the cholera pathogenesis study, both *in vivo* and *in vitro* colonization assays were performed and the colonization efficiency of the bacteria in both methods were compared. The results showed that the colonization of *V. cholerae* vaccine candidates in the *in vitro* colonization method is more efficient than the *in vivo* method.

CHAPTER 1

INTRODUCTION

1.1 *Vibrio cholerae* and its structure

Vibrios are a group of Gram-negative, curved or straight motile rods that are normally found in the aquatic environment (Uma *et al.*, 2003). Robert Koch obtained pure cultures of *Vibrio cholerae* and stated that *V. cholerae* was a little bent microorganism that resembled a comma or a spiral and it was highly motile with a single polar flagellum on gelatine plates. Besides, *V. cholerae* is a non-spore forming bacterium and possess filamentous pili that form bundles on the bacterial surface. It belongs to a family of pili whose chemical structure is similar to those of the gonococcus, and a number of other bacterial pathogens. (Thompson *et al.*, 2004, Angelichio *et al.*, 1999, Guentzel and Berry, 1975).

Several tests have been carried out to determine the biochemical properties of *V. cholerae.* This bacterium can grow on marine agar and on thiosulfate-citrate-bile salt-sucrose agar (TCBS) selective medium. TCBS is an ideal for the selective isolation and purification of *V. cholerae*, utilizes sucrose and forms yellow colonies on this medium. *V. cholerae* can grow in aerobic or anaerobic conditions. This bacterium also shows oxidase positive reaction. Besides, *V. cholerae* has a low tolerance for acid, but can grow well in alkaline (pH8.0-9.5) conditions which inhibit many other Gram-negative bacteria. *V. cholerae* is distinguished from other vibrios by its biochemical reactions,

lipopolysaccharide (LPS) O antigenic structure, and production of cholera toxin (CT). There is over 200 recognized *Vibrio cholerae* O antigen serogroups, essentially only O1 and O139 can cause severe cholera (Liang *et al.*, 2003, Uma *et al.*, 2003, Nesper *et al.*, 2002).

V. cholerae as a species includes both pathogenic and non-pathogenic strains that vary in their virulence gene content. This bacterium is thought to contain a wide variety of strains and biotypes. It might receive and transfer genes for toxins, colonization factors, antibiotic resistance, capsular polysaccharides that provide resistance to chlorine and new surface antigens, such as the O139 lipopolysaccharide and O antigen capsule. Many genes associated with virulence in cholera are known to have been acquired through gene-transfer events that have mediated the transformation of avirulent strains to those capable of causing outbreaks of diarrhea (Uma *et al.*, 2003). The lateral or horizontal transfer of these virulence genes by phage, pathogenicity islands inside the gene and other accessory genetic elements provide insights into how bacterial pathogens emerge and evolve to become new strains (Heidelberg *et al.*, 2000).

1.2 Nomenclature

In the past, a wide variety of Gram-negative and rod-shaped bacteria with polar flagella were classified under the genus *Vibrio* (Faruque *et al.*, 1998). However, *V. cholerae* is further classified into serogroups based on its somatic antigen, which is known as O antigens (Uma *et al.*, 2003). Serogroup O1 was supposed to include all the strains that were responsible for epidemic and endemic cholera. It has three serotypes which include Inaba, Ogawa and Hikojima. The Hikojima serotype was rarely reported. These serotypes can be further distinguished into two biotypes, namely classical and El Tor based on biochemical properties, phenotypic differences and susceptibility to bacteriophages (Liang *et al.*, 2003, Uma *et al.*, 2003, Lin *et al.*, 1999, Faruque *et al.*, 1998, Nair *et al.*, 1994).

Some known serogroups of non-O1 vibrios was also reported. These vibrios possess biochemical and morphological characteristics very similar to those of the cholera vibrio but are nonagglutinable with polyvalent O1 antiserum. These vibrios are only agglutinable with their own antisera. Usually, non-O1 serogroups of *V. cholerae* had been associated mostly with sporadic cases of diarrhea and extraintestinal infections until 1993, when a large cholera-like outbreak in Bangladesh and India (Uma *et al.*, 2003) was found to be caused by a *Vibrio cholerae* non-O1 strain. This organism did not belong to any of the known O serogroups of *V. cholerae* but to a new serogroup, which was later designated as O139. Since then, *Vibrio cholerae* O139 has been persisting as a second etiologic agent of cholera (Faruque *et al.*, 1998, Nair *et al.*, 1994). Until today, the *Vibrio cholerae* El Tor biotype is still the main pathogen causing epidemics and isolated cases of cholera (Liang *et al.*, 2003, Uma *et al.*, 2003).

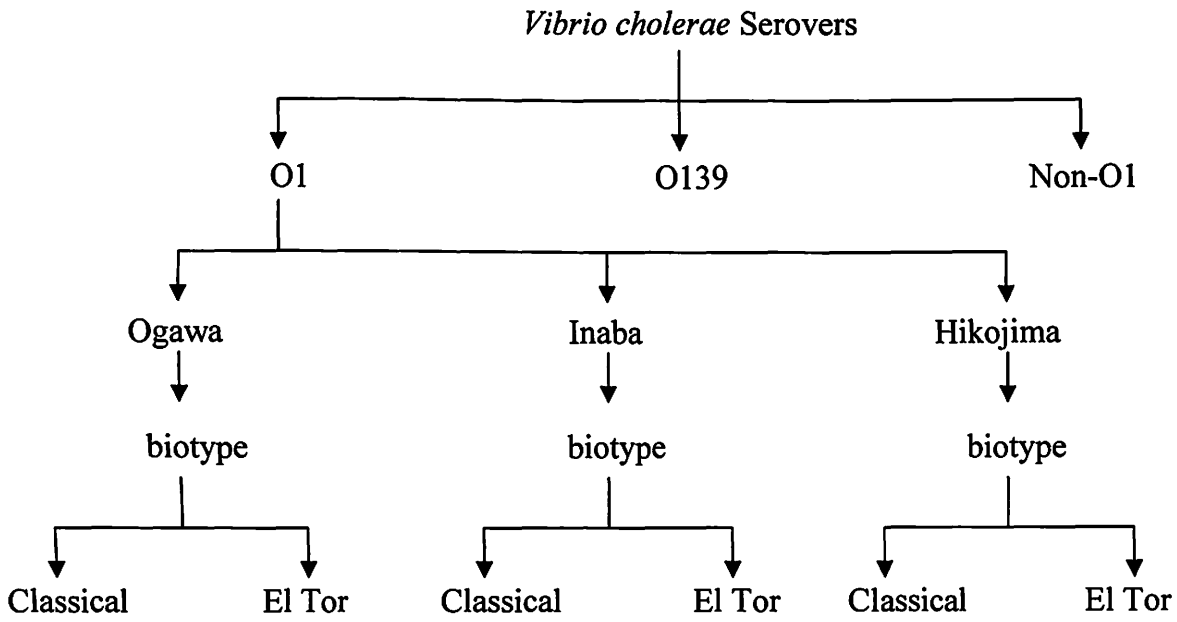

Figure 1.2.1: Schematic diagram of *Vibrio cholerae* classification

1.3 *Vibrio cholerae* and its epidemiology

Cholera is a severely dehydrating diarrheal condition and this disease is epidemic, endemic and pandemic in nature. The epidemic forms of the disease are caused by the O1 and O139 serogroups of *Vibrio cholerae* (Uma *et al.*, 2003).

Epidemic cholera is spread primarily through contaminated water under conditions of poor sanitation, particularly where sewage treatment is absent or defective. The disease is now endemic, claiming thousands of lives every year, of which the vast majority occur in children (Faruque *et al.*, 1998). Hallmarks of the epidemiology of cholera include a high degree of clustering of cases by location and season, highest rates of infection among children between 1 to 5 years of age in areas of endemic infection, antibiotic resistance patterns that frequently change from year to year and clonal diversity of epidemic strains (Faruque *et al.*, 1998).

Cholera can cause the death of a healthy adult within hours of onset (Lencer, 2001). Eight worldwide pandemics have been documented since the mid-nineteenth century and each pandemic lasting 5 to 25 years (Faruque *et al.*, 1998). Cholera is endemic in the Indian subcontinent and Africa. Over the past two centuries, it spread beyond this historic locale to other parts of Asia, Indonesia, and Europe (Faruque *et al.*, 1998). Besides, cholera is also endemic in southern Asia and parts of Africa (Chow *et al.*, 2001) as well as Latin America, where seasonal outbreaks occur widely and are particularly associated with poverty and poor sanitation. Cholera has been epidemic in southern Asia for at least 1,000 years, and has also spread worldwide to cause eight pandemics since 1817 (Heidelberg *et al.*, 2000).

A distinctive epidemiological feature of cholera is its appearance in a regular seasonal pattern in areas of endemic infection and in explosive outbreaks starting in several distinct foci simultaneously, indicating possible role of environmental factors in triggering the epidemic process (Faruque *et al.*, 1998). Environmental and climactic factors have been shown to contribute to the epidemic spread of *V. cholerae*. In areas where it is endemic, cholera occurs in recurrent peaks at the end of the monsoon season. These peaks have been linked to water temperature changes and zooplankton blooms as well as to the prevalence of vibriophages in the environment (Alam *et al.*, 2005). Thus, cholera has been categorized as one of the "emerging and reemerging infections" threatening many developing countries.

However, according to Nair *et al.* (1994), the pandemic potential of the *Vibrio cholerae* O139 serogroup is obvious with the spread of this serogroup to several countries in the Asian and neighboring subcontinents, including Thailand and Pakistan. Furthermore, imported cases of O139 cholera have been reported from the United States, United Kingdom, Singapore, Switzerland, Germany, and Japan.

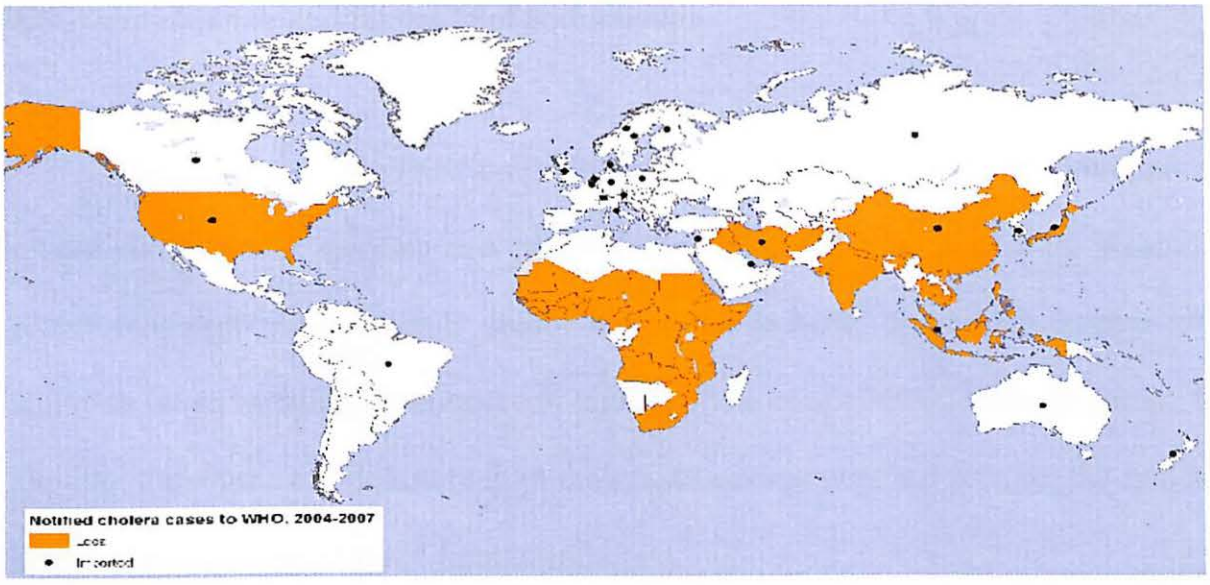

Figure 1.3.1: Countries/areas with cholera cases from 2004-2007 (WHO, 2007)

1.4 Ecology of *Vibrio cholerae*

V. cholerae is a gram-negative bacterium that naturally inhabits the aquatic environment and infects human beings (Uma *et al.*, 2003). It is the causative agent of cholera, a devastating diarrheal disease that affects millions of people in the world each year (Hsiao *et al.*, 2006, Chakraborty *et al.*, 2001). *V. cholerae* has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (Faruque *et al.*, 2004, Faruque *et al.*, 1998), including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Thompson *et al.*, 2004, Kierek and Watnick, 2003, Heidelberg *et al.*, 2000). Besides, *Vibrio cholerae* is normally found in association with planktonic species in surface water (Hsiao *et al.*, 2006, Merrell *et al.*, 2000). However, virulent *Vibrio cholerae* now lurks in coastal waters throughout the hemisphere and in the drinking water of locales with poor sanitation. Thus, water is clearly a vehicle for transmission of *Vibrio cholerae (Faruque et al., 2004).*

1.5 *Vibrio cholerae* and the mode of transmission

The pathogenic bacterial strains are able to colonize human small intestine and release cholera toxin, resulting in a secretory diarrhea that can be fatal in the absence of proper treatment. *V. cholerae* is unique among the bacterial diarrheal pathogens in its ability to cause worldwide pandemics of disease (Alam *et al.*, 2005). Thus, the factors that facilitate the organism's departure from the aquatic environment and promote the epidemic spread of human disease are of great interest.

Vibrios are ubiquitous in aquatic settings and that many forms of vibrios are non-pathogenic for human. However, some strains of *V. cholerae* infect human and cholera is one of the major causes of morbidity and mortality in developing and underdeveloped countries (Chakraborty *et al.*, 2001). This disease is spread through contaminated water and food (Sheahan *et al.*, 2004, Thompson *et al.*, 2004, Merrell *et al.*, 2000). According to the report of World Health Organization (WHO), the sudden large outbreaks of this disease are usually caused by a contaminated water supply. The cholera that is transmitted by direct person-to-person contact rarely occurs. In highly endemic areas, it is mainly a disease of young children and it rarely affects breastfeeding infants.

V. cholerae is often found in the aquatic environment and is part of the normal flora of brackish water and estuaries. It is often associated with algae blooms (plankton), which are influenced by the temperature of the water. However, cholera may transmit through its vectors which include zooplankton such as copepods, chironomid insects, and cyanobacteria (Thompson *et al.*, 2004). Naturally, human beings are also one of the reservoirs of the pathogenic form of *V. cholerae* (Merrell *et al.*, 2000).

1.6 *Vibrio cholerae* and its pathogenesis

Cholera is the consequence of the action of toxin produced by *Vibrio cholerae* in the small intestine of humans. The toxin has been isolated and characterized, and its mode of action, activation of host adenylate cyclase and consequent hypersecretion of electrolytes and water, is reasonably well understood (Nelson *et al.*, 1976). In human cholera, infecting *V. cholerae* is confined to the mucosa and lumen of the intestinal tract. This bacterium may possess a mechanism which confers upon the cells the capacity to attach to and readily proliferate on the surface of the intestinal epithelium of infected hosts (Guentzel and Berry, 1975). The organisms elaborate a protein enterotoxin which mediates the net fluid secretion manifested as acute diarrhea. Fluid loss originates entirely in the small intestines and primarily in the jejunum (Baselski *et al.*, 1978).

To produce disease, *V. cholerae* must reach the small intestine in sufficient numbers to multiply and colonize. Colonization of the entire intestinal tract from the jejunum to the colon by *V. cholerae* requires organism adherence to the epithelial surface. *V. cholerae* moves along and attaches to surfaces with the aid of the flagellum and pili, which act as adhesins. This bacterium forms microcolonies on surface and subsequently produces exopolysaccharides, which stabilize the pillars of the biofilm (Thompson *et al.*, 2004). The outstanding feature of *V. cholerae* pathogenicity is the ability of virulent strains to secrete cholera toxin (CT), which is responsible for the disease cholera (Merrell *et al.*, 2000, Faruque *et al.*, 1998). CT causes water and electrolyte shift from the cell to the intestinal lumen which is the fundamental cause of watery diarrhea of cholera. Thus, cholera pathogenesis relies on the synergistic effect of a number of pathogenic factors produced by

toxigenic *V. cholerae* (Faruque *et al.*, 1998) such as the factors that produced by CTX element and TCP pathogenicity island in the gene.

In general, the important steps of infection include ingestion of *V. cholerae* along with contaminated food or water, passage through the gastric acid barrier of the stomach, penetration through the intestinal mucus lining, adherence to intestinal epithelial cells, multiplication, and the production of cholera toxin (Nesper *et al.*, 2002). Finally, the action of the cholera toxin will cause the disease on the victims.

1.7 Clinical aspects of cholera disease

Cholera is characterized by a severe watery diarrhea caused by toxigenic bacterium *V. cholerae*, which colonizes the small intestine and produces an enterotoxin which is known as cholera toxin (Faruque *et al.*, 1998). Typical cholera has a rapid onset, beginning with abdominal fullness and discomfort, rushes of peristalsis, and loose stools. Vomiting may also occur. The stools quickly become watery, voluminous, almost odorless, and contain mucus flecks, giving it an appearance called rice-water stools. Clinical features of cholera result from the extensive fluid loss and electrolyte imbalance, which can lead to extreme dehydration, hypotension, and death within hours if untreated.

1.8 Prevention and control of cholera disease

Epidemic cholera is a disease of poor sanitation and water supply. It does not persist where treatment and disposal of human waste is adequate. When cholera appears in a community, it is important to ensure that hygienic disposal of human faeces, an adequate supply of clean drinking water, and good food hygiene. Cholera disease can be prevented by drinking boiled water. Since good sanitary conditions do not exist in many parts of the world, secondary local measures such as boiling or chlorination of water during epidemics are required. Besides, the water supply systems should incorporate filtration of drinking water in order to remove the bacteria.

Effective food hygiene measures include cooking food thoroughly and eating it while still hot. Besides, to prevent the infection, people are advised to avoid cooked foods from being contaminated by contact with raw foods, contaminated surfaces or flies. Consumption of raw fruits should be reduced, unless they are first peeled. The cases associated with crustaceans can be prevented by adequate cooking.

The proper sanitation management program to ensure supply of uncontaminated water is one of the means to prevent the outbreak of the cholera disease (Reidl and Klose, 2002). The essential features of a national diarrhea diseases control programme include a national epidemic control committee, a well-established surveillance system, environmental sanitation and safe water supplies, health education, and hands-on training in clinical management should be emphasized. Since cholera is a water-borne disease, environmental monitoring for the presence of *V. cholerae* strains with pathogenic potential is also

important to identify the source of strains causing either epidemics of cholera or sporadic cases of cholera (Faruque *et al.*, 2004).

Vaccination against cholera is a powerful and feasible disease prevention strategy because recovery from infection results in long-term protective immunity (Liang *et al.*, 2003). Vaccines prepared from killed or live attenuated vibrio strains have the potential to stimulate the local IgA immune response. The first live attenuated vaccine developed for *V. cholera* was CVD103-HgR that was designated for O1 serogroup (Favre *et al.*, 1996). Later, Peru-15 was also produced as a vaccine candidate for O1 serogroup (Qadri *et al.*, 2005).

1.9 Background of *Vibrio cholerae*

In 1854, *Vibrio cholerae* was discovered by Filippo Pacini, an Italian physician. He identified *V. cholerae* as the causative agent of cholera while studying the outbreak of this disease in Florence. He further pointed out that cholera was a contagious disease after he examined the intestinal mucosa of fatal victims of cholera by using microscope and detected *Vibrio cholerae* in all samples (Thompson *et al.*, 2004). Toxigenic strain of the Gram-negative bacterium *V. cholerae* causes a life-threatening diarrheal disease that can kill its victims within hours of the onset of symptoms.

Prior to 1992, two biotypes of *Vibrio cholerae* serogroup O1, classical and El Tor, were responsible for all epidemic cholera (Jouravleva *et al.*, 1998). The vibrio responsible for the seventh pandemic is known as *Vibrio cholerae* O1, biotype El Tor. This bacterium

is responsible for Asiatic or epidemic cholera and it is a well-recognized cause of morbidity and mortality throughout the world (Johnson *et al.*, 1994). The seventh pandemic began in 1961 when the vibrio first appeared as a cause of epidemic cholera in Celebes (Sulawesi), Indonesia. The disease then spread rapidly to other countries of eastern Asia and reached Bangladesh in 1963, India in 1964, and the USSR, Iran and Iraq in 1965-1966. In 1970 cholera invaded West Africa and in 1991 cholera struck Latin America.

Initial reports stated that until 1992, only *Vibrio cholerae* serogroup O1 caused epidemic cholera. Some other serogroups could cause sporadic cases of diarrhea, but not epidemic cholera. Late in 1992, large outbreaks of cholera occurred in southern and eastern India as well as southern Bangladesh (Johnson *et al.*, 1994) that were caused by a previously unrecognized serogroup of *V. cholerae*, and then designated as O139, synonymous with the Bengal strain. Since then, O139 outbreaks have occurred in several countries in Southeast Asia (Nesper *et al.*, 2002, Nair *et al.*, 1994). However, beginning in 1994, O1 El Tor strains have re-emerged as the predominant cholera causing organisms on the Indian subcontinent, although O139 strains continue to co-exist (Nesper *et al.*, 2002).

Vibrio cholerae O1 and O139 are commonly known to carry a set of virulence genes necessary for pathogenesis in humans. Recent studies have indicated that virulence genes or their homologues are also dispersed among environmental strains of *V. cholerae* belonging to diverse serogroups that appear to constitute an environmental reservoir of virulence genes. Although the roles of virulence-associated factors in the environment and the selection pressures for environmental *V. cholerae* carrying virulence genes is not clear, it is possible that these strains may be precursors of pathogenic strains or may participate in

gene transfer events leading to the origination of pathogenic strains (Faruque *et al.*, 2004). Many genes associated with virulence in cholera are now known to have been acquired through gene-transfer events that have mediated the transformation of avirulent strains to those capable of causing outbreaks of diarrhea (Uma *et al.*, 2003). The acquisition of key virulence genes by horizontal transfer events is important in the evolution of pathogenic strains of *V. cholerae*. Such a genetic transfer event preceded the 1992 emergence of a novel serogroup of epidemic cholera, *Vibrio cholerae* O139, which arose through the acquisition of the *wbf* gene cluster from a nontoxigenic *V. cholerae* isolate (Alam *et al.*, 2005).

The appearance of the O139 serogroup, which shares ominous similarities with that of the O1 serogroup, has several implications. For instance, until recently, only O1 *Vibrio cholerae* strains were considered to cause cholera while the non-O1 *Vibrio cholerae* strains and other enteric pathogens caused only a cholera-like infection. However, G. Balakrish Nair (1994) proposed that the disease caused by O139 strains should be designated as cholera and should be a disease notifiable to the World Health Organization because of the similarity in the clinical profile of the disease and because of the epidemic potential of the O139 serogroup, which is identical to that of the O1 serogroup (Nair *et al.*, 1994).

From previous studies, several lines of evidence suggested that the O139 serogroup closely resembles the O1 El Tor biotype. However, there are also several differences between O139 and O1 serogroups. Existing data suggest that serogroup O139, synonymous with Bengal arose from a serogroup O1 biotype El Tor, lack the O1-specific antigen. So, non-agglutinability of the strains with O1 antiserum was the main differences between